IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/759,099	Confirmation No. 4916
Applicant	:	Timothy J. O'Leary et al.	
Filed	:	January 20, 2004	
TC/A.U.	:	1637	
Examiner	:	CALAMITA, H.	
Docket No.	:	AFIP 03-16 01	
Customer No.	:	27370	

For: IMMUNOLIPOSOME-NUCLEIC ACID AMPLIFICATION (ILNAA) ASSAY

COMBINED DECLARATION UNDER 37 C.F.R. 1.131 AND 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

We, Dr. Jeffery Mason and Dr. Timothy O'Leary, hereby declare and state that:

1. We are inventors of the above patent application.

2. We are familiar with the rejection of the pending claims, particularly over Singh et al. (Anal. Chem., November 2000) in view of Wu et al. (Letters in Applied Microbiology, May 2001).

3. These references were discussed in detail at a June 12, 2007 personal Examiner interview attended by us, our representative, Examiner Calamita, and Primary Examiner Strzelecka.

4. The present application is directed to an Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay which includes encapsulating a plurality of identical nucleic acid segments within closed shell liposomal bilayers; associating receptors to the extravesicular surface of said liposomal bilayers; exposing the receptors to an immobilized target analyte, which binds to the liposomal bilayer associated receptors; removing unbound liposomal bilayers; lysing the bound liposomal bilayers to release the

nucleic acid segments; amplifying the nucleic acid segments released from the liposomal bilayers, and detecting the amplification products of the nucleic acid segments to quantify the amount of the target analyte.

I. 1.131 Declaration: Antedating the Singh et al. and Wu et al. References

5. We set forth the following facts to antedate the Singh et al. and Wu et al. references.

6. Attached hereto are 2 redacted letters concerning a grant application for Sensitive Detection of Antigens or Antibodies by Immunoliposome - DNA Amplification Hybrids that was submitted to the US Army Medical Research Institute of Infections Diseases, Fort Detrick, MD. The grant was ultimately not funded. Both letters are dated prior to November 2000. See Exhibits 1-2.

7. A partial redacted copy of the corresponding grant application is also attached. See Exhibit 3.

8. Also attached is a partial copy of the Armed Forces Institute of Pathology internal annual report, which on page 64 under RESEARCH indicates the ongoing "developmental work in immunoliposome-PCR (an ultrasensitive assay system for biological and chemical antigens)". The annual report is dated prior to November 2000. See Exhibit 4.

9. Beginning prior to the November 2000, we and our team diligently worked to reduce the immunoliposome-nucleic acid amplification (ILNAA) assay to practice.

10. As evidenced by the attached Letter of Intent to Mr. Glisson of the U.S. Army Medical Research and Materiel Command dated 20 April 2001, research was ongoing. In the Letter of Intent, Dr. Mason describes a proposal for coupling the selectivity and high binding affinity of antibodies for biological molecules with the near-infinite signal amplification of PCR by encapsulating short DNA sequences (primers) into enclosed

phospholipid membranes (liposomes) and conjugating antibodies specific to biological hazards to the outer liposomal membranes. See Exhibit 5.

11. Research Proposal BAA 99-1 followed the Letter of Intent a month later. See Abstract on page 2 of Exhibit 6.

12. We continued to work on the invention as part of our normal duties for the U.S. Army. At no time did we abandon or stop working on the ILNAA assay other than taking care of other matters that are part of our normal work routines. Our work continued until a working method was completed and reduced to practice in September 2002.

13. After completing a working method, we prepared a Department of the Army Invention Disclosure to advise the U.S. Army of the invention, a partial redacted copy of which is attached. The description of the invention and drawings confirm that we and our team reduced the invention to practice prior to January 10, 2003. See Exhibit 7.

14. The individuals on our team can provide further corroboration that for the period prior to November 2000 and until completion of a working example in September 2002, we were diligent in pursuing reduction to practice of the invention and at no time ceased working on the project or otherwise stopped our efforts during the course of our normal workday.

15. All activities and disclosures described above occurred in the United States of America.

II. 1.132 Declaration

A. The Liposomes of Singh et al. Are Incompatible With DNA Encapsulation

16. Singh et al. uses a lipid mixture (L-α-distearoylphosphatidycholine) and L-α-dimyristoylphosphatidylehtanolamine) that is not compatible with DNA encapsulation.

The extrusion method of Singh et al. for forming liposomes is also not compatible with DNA encapsulation. The lipid mixture and extrusion method only allow for passive encapsulation of DNA segments (amplicons) into the liposomes.

17. There are approximately 220,000 lipid molecules per liposome for the 120 nm-diameter liposomes formed using the membrane extrusion method of Singh et al. Thus, the total lipid concentration yields about 85×10^{12} liposomes in 1 mL of the aqueous buffer. This concentration of liposomes (of bilayer thickness 3.7 nm) yields a total internal volume of about 7.5×10^{-16} mL. The liposomes formed by the method of Singh et al. use the membrane extrusion method, which would passively encapsulate the amount of amplicon present in the solution as the liposomes form. Accordingly, the number of moles of amplicon encapsulated into each liposome would be about 7×10^{-24} moles. This corresponds to only about 4 amplicons per liposome.

18. Four (4) amplicons per liposome is unacceptable for use in the ILNAA assay for the following reasons:

(a) only 6% of the total amplicon in solution will be encapsulated into the liposomes, with 94% being on the outside and lost during the purification of the liposomes; and

(b) 4 amplicons per liposome places the distribution of amplicons in the liposomes into the realm of Poisson statistics. More specifically, for 100 liposomes, one would expect almost 25% of the liposomes to be empty. This is unacceptable for the proper performance of the ILNAA assay. To avoid complications resulting from Poisson statistics, there ideally should be at least 100 amplicons per liposome.

19. In summary, the method of Singh et al. is unsatisfactory for preparing liposomes with encapsulated DNA amplicons for use in the ILNAA assay method. Further, one or ordinary skill in the art would not be able to simply substitute the

rohamine-labeled fluorophores of Singh et al. with the DNA segments of Wu et al. as asserted by the Examiner.

B. Superior Results Obtained With the Claimed ILNAA Assay

20. The present ILNAA application teaches an Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay for achieving a detection limit of 10-1000 molecules of toxin in subattomolar quantites (i.e., below 1×10^{-18} moles). In fact, the immunoassay method of the present invention can achieve a detection limit of 10 molecules of cholera toxin (17×10^{-24} moles or 17 yoctomoles) and 12 molecules of botulinum neurotoxin type A (20 yoctomoles).

21. The method of Singh et al. teaches an assay method with a detection limit of 1×10^{-10} moles or 0.1 nanomoles for botulinum neurotoxin (page 6024).

22. The method of Wu et al. teaches an assay with a detection limit of 33×10^{-18} moles or 18 attomoles for botulinum neurotoxin type A (Abstract).

23. As shown in the Table below, the Immunoliposome - Nucleic Acid Amplification (ILNAA) Assay of the present invention is 5×10^{12} times more sensitive than the method of Singh et al. and 2×10^{6} times more sensitive than the method of Wu et al.

TABLE: COMPARISON OF DETECTION LIMITS

	Detection Limit of Botulinum neurotoxin	Difference In Detection Limit Between ILNAA application and Prior Art
Present Application	20×10^{-24} moles	----
Singh et al.	1×10^{-10} moles	5×10^{12} ILNAA is 5 Trillion (5,000,000,000,000) Times more sensitive
Wu et al.	33×10^{-18} moles	2×10^{6} ILNAA is 2 Million (2,000,000) Times more sensitive

24. The dramatic increase in sensitivity achieved by the method of the present patent application demonstrates the superior properties of the claimed methods over either the fluoroimmunoassay of Singh et al. or the immuno-PCR assay of Wu et al.

25. In addition, the superior sensitivity of the claimed ILNAA cannot be achieved by the collective teachings of Singh et al. and Wu et al. As noted, DNA segments are not compatible with the liposomes of Singh et al. Further, even if these references were properly combinable, which they are not, one of ordinary skill in the art would have at best estimated that any combination would have increased the sensitivity of the Singh et al. assay to be close to the attomolar level (10^{-18}) of Wu et al., nowhere close to the yoctomolar (10^{-24}) level achieved by the present invention.

IV. VERIFICATION

26. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false

statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Jeffrey G Mason

Dr. Jeffrey Mason — 11 February 2008 Date

Timothy J. O'Leary

Dr. Timothy J. O'Leary — 11 February 2008 Date

EXHIBIT 1

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORT DETRICK, FREDERICK, MARYLAND 21702-5011

REPLY TO ATTENTION OF:
Extramural Research Office

Timothy J. O'Leary, M.D., Ph.D.
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Dear Doctor O'Leary:

Your proposal entitled "Sensitive Detection of Antigens or Antibodies by Immunoliposome - DNA Amplification Hybrids", Log No. 92303001 was technically reviewed and will be considered for award. However, in addition to a favorable technical review, a final decision to award is contingent upon such factors as availability of funds, final prioritization of programmatic needs, and negotiation of costs, terms, and conditions. The formal and final notification of contract award will only be made by a Contracting Officer and you should not use this letter to support incurring any costs associated with this work.

To complete processing your proposal for possible future funding, a Memorandum of Environmental and Safety Analysis, Broad Agency Announcement, Appendix G, is required. Enclosed is a copy of the current Announcement for your convenience. Questions concerning safety and environmental issues should be directed to USAMRDC, SGRD-SF, Fort Detrick, Frederick, MD 21702-5012, Phone (301) 619-2003.

This communication is not a commitment on the part of this Command to award a contract to your organization. You will be contacted by a representative of USAMRDC's Acquisition Division once a decision has been made to fund your proposal and funds are available. The award of any contract usually requires a minimum of five months from this notification.

Thank you for your interest in our Biological Defense Research Program and for the opportunity to review your proposal.

Sincerely,

Robert R. Rosato, Ph.D.
Extramural Rsch Programs

Copies Furnished:
Acquisition Management Office
Medical Biological Defense Research Program
Reviewers

EXHIBIT 2

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORT DETRICK, FREDERICK, MARYLAND 21702-5011

REPLY TO ATTENTION OF:
Extramural Research Office

Timothy J. O'Leary, M.D., Ph.D.
Armed Forces Institute of Pathology
Washington, DC 20306-6000

Dear Doctor O'Leary:

Since my letter to you of our mission and funding priorities have changed. I am sorry to inform you that we will not be able to provide support for your proposal "Sensitive Detection of Antigens or Antibodies by Immunoliposome - DNA Amplification Hybrids", Log No. 92303001.

We held your proposal in the hope that programmatic issues would be resolved and that support from our limited extramural budget could be made available.

I will retain your proposal for possible future consideration should our requirements and budget projections change.

Thank you for your interest in our Medical Biological Development Research Program and the opportunity to review your proposal.

Sincerely,

Robert R. Rosato, Ph.D.
Extramural Research Programs

Copies Furnished:
Acquisition Management Office
Medical Biological Defense Research Program
Reviewers

EXHIBIT 3

Table of Contents

BODY OF PROPOSAL

OBJECTIVE: To develop more sensitive and rapid methods for detecting, identifying, and quantitating military disease hazards, particularly botulinum toxin.

INTRODUCTION

The early and rapid identification of military disease hazards is critically important to the protection of military personnel deployed in potential combat situations. Identification of infectious diseases and chemical and biological agents are all important. Current methods of rapidly identifying infectious agents include immunodetection methods and methods based upon amplification of nucleic acid sequences, such as PCR. Immunodetection methods lack sensitivity for very low numbers of organisms or antigens, however, and PCR-based methods typically take up to a day to complete. Immunodetection methods can also be used for identifying many biological agents and some chemical agents; however, the sensitivity limitations remain and the methods have little practical use in identifying important military disease hazards, such as botulinas toxin, which are present in extremely low concentrations. Nucleic acid amplification methods are not useful for these detection problems.

Recently Sano *et al* (1993) demonstrated a method for using polymerase chain reaction amplification of DNA as a detection system for antigens. This method depends upon coupling specific antibodies to DNA sequences, which are then amplified only if the antigen is present. The method is very enticing, but presents several difficulties for military application. The first of these difficulties is that the detection sensitivity is still significantly limited. Detection of only a few copies of a DNA molecule by polymerase chain reaction is often difficult, especially in the presence of *Taq* polymerase inhibitors which are present in many samples. Secondly, as mentioned above, to carry out many cycles of amplification by PCR is time consuming.

We have recently conceived a method which overcomes both of these problems. Like the Sano *et al* (1993) method, DNA amplification is used to enhance the sensitivity of detecting an antigen or an antibody. The limitations due to the one to one correspondence between DNA and antigen are resolved by incorporation of target DNA into immunoliposomes. As a result, fewer cycles of DNA amplification are required to detect antigen; these are carried out in a capillary thermal cycler which is capable of going through each amplification step in less than one minute. Subsequent detection of DNA is carried out through a rapid colorimetric method.

We propose to study the use of this immunoliposome - DNA amplification hybrid method for detecting low levels of botulinum toxin. The method will be compared with simple immunologic detection, as well as with the method proposed by Sano, Smith and Cantor (1).

EXPERIMENTAL DESIGN

We will compare the use of ELISA methods, the immunoliposome method outlied above, and the Sano *et al* method for detecting botulinas toxoid. The toxoid will be utilized instead of native toxin to obviate the difficulties necessary in handling the toxin in the early stages of investigation. Both toxoid and antibodies to be used in the study will be supplied by Major Korch of the US Army Research Institute of Infectious Diseases.

In year one, we propose to establish the feasibility of the assays for botulinum toxin. Following feasibility demonstration, we will attempt to optimize the assay by developing strategies for reducing nonspecific binding of the immunoliposomes, and incorporating an optimal amount of reporter DNA into the immunoliposomes. In year two we will continue to optimize the general form of the assay, particularly by exploring the use of alternative detection techniques and amplification techniques other than PCR. In addition, we will begin to explore the use of the method for the detection of other pathogens of military relevance, including Leishmania, E. coli, and a toxin to be determined jointly by AMRDC and the investigators. Year three will be devoted to efforts to frabricate prototypical field-deployable systems and to validate the use of the techniques on the types of specimens likely to be encountered during deployment. At each phase of the work, comparisons between this system and existing systems for detecting pathogens of military significance will be compared from the standpoints of sensitivity, specificity, and ease of use.

Since botulinum toxin is to be assayed as free ligand, the first step required in each of these assays is to capture the toxin using an antibody immobilized in a microtitre well. This will be accomplished using one of the mouse monoclonal antibodies supplied by Major Korch, using standard methods. Differences in assay efficacy will therefore reflect the detection method, rather than the capture method, as an identical capture system will be used for each assay method.

I. Immunoliposome Method

A. Principle: Antigen which is immobilized in a microtiter well may be detected by the following sequence:

EXHIBIT 4

DEPARTMENT OF CELLULAR PATHOLOGY

Timothy J. O'Leary, M.D., Ph.D.
Chairperson

STAFF

Danny Urquhart, Research Administrator
Anna Thomas, Secretary

In addition to his usual administrative responsibilities, Dr. O'Leary serves on the editorial board of two journals, serves as the chairholder of the National Committee on Clinical Laboratory Standards subcommittees on molecular hematology and immunocytochemistry, as vice-chairholder of the NCCLS Molecular Methods Area Committee, as consultant to the Food and Drug Administration, as attending pathologist at the National Institutes of Health, and as director for the USCAP course "Tumor Prognosis." He lectures frequently at national and international courses, conferences, and symposia.

One major administrative/scientific accomplishment has been in the area of funding. Departmental funding has increased from zero in 1987, to almost $1 million this year. Research and educational activities include the use of classical, quantitative, and molecular methods to predict tumor prognosis and research on the structure of biological membranes. Dr. O'Leary participates in the diagnostic activities of the Cytopathology and Molecular Pathology Divisions.

QUANTITATIVE PATHOLOGY

Robert L. Becker, Col, USAF, MC, Associate Chairperson and Chief, Quantitative Pathology
William Oliver, LTC, MC, USA, Forensic Pathlogist
Ulrika Mikel, M.G.A., Cytologist
Nina Sweeney, M.S.E.E., Computer Scientist
Lena Hohmann, M. Pharm, Biologist
Joseph Griffin, Ph.D., Biologist
Annette Geissel, M.S., Biologist
Elzbieta Kaczmarek, Ph.D., Callender-Binford Fellow
Ruixia Zhao, Computer Scientist

The division's responsibilities include flow cytometry (FCM) and image analysis. FCM is staffed by Dr. Griffin with help from Ms. Geissel and Ms. Hohmann and primarily involves ploidy and S-phase analysis of paraffin-embedded tissue for consultation and research. Other staff have a broad mission to develop and apply computer-assisted techniques extracting information from pathology images Activities include quantitative microscopy, forensic image analysis, artificial intelligence, and scientific computing. Applications in practice and under development include image analysis of cell ploidy and growth, patterned injury analysis, and three-dimensional visualization/analysis of cells, tissues, and forensic images. The division has performed seminal work in three-dimensional visualization of thick tissue sections and in forensic image analysis, resulting in formal commendation by senior military commanders and by the Attorney General of the United States. Division staff are extensively tasked to assist development of computer-oriented imaging applications throughout the Institute.

particularly those involving wide-area networking.

CYTOPATHOLOGY

M. Tellado, LtCol, USAF, MC, Cytopathologist and Acting Chief, Cytopathology
S. Buckner, M.S., Senior Cytotechnologist, Consultation Service
I. Ali, M.S., Chief, Cytotechnologist, Screening Laboratory
E. Delgado, M.S., Senior Cytotechnologist, Screening Laboratory
R. McNeill, M.S., Senior Cytotechnologist, Screening Laboratory
A. Stevens, M.S., Junior Cytotechnologist, Screening Laboratory
C. Brooker, Msgt, USAF, Superintendent, Accessioning and Quality Assurance
P. Edgar, Secretary
A. Reeder, Accessioning technician
R. Matthres, Accessioning technician

Work includes consultation on difficult cases, primary screening of approximately 40,000 cases per year (primarily Air Force, but also Navy and PHS), research on automated rescreening and telecytology, training for military and PHS residents, an annual course, and a joint cytopathology fellowship program with the Johns Hopkins University.

Cytologic examination is increasingly important as a definitive diagnosis technique; development of a strong, nationally recognized presence in cytopathology is the focus of the department's most intensive developmental activity.

BIOPHYSICS

J. Mason, Ph.D., Chief, Biophysics
M. Batenjany, Ph.D., Postdoctoral Fellow
R. Cunningham, M.S., Chief, Flow Cytometry Laboratory

Work includes basic research on membrane structure and the effects of toxins on membranes and membrane proteins, and use of biophysical methods for pathologic diagnosis. Highlights include the development of immunoliposomal PCR and the development of preparation methods for infrared and Raman microspectroscopy of tissue, which have now been adopted worldwide.

MOLECULAR PATHOLOGY

J. Taubenberger, M.D., Ph.D., Chief, Division of Molecular Pathology
J. Lichy, M.D., Ph.D., Director, Molecular Diagnostics Laboratory
A. Krafft, Ph.D., Chief Medical Technologist
B. Duncan, B.S., Medical Technologist
H. Diebert, M.T., Medical Technologist
C. Wright, Ph.D., Senior Investigator
T. Fanning, Ph.D., Senior Investigator
A. Tatro, Ph.D., Postdoctoral Fellow
A. Hubbs, Ph.D., Postdoctoral Fellow
G. Brown-Stephano, Ph.D., Postdoctoral Fellow
A. Reid, M.S., Biologist
M. Tsai, M.S., Biologist
M. Zavar, B.S., Biologist, ARP
M. Majidi, M.S., Biologist, ARP

The major diagnostic activity is operation of the Molecular Diagnostics Laboratory. This laboratory will conduct approximately 6,000 assays on 600 formalin-fixed, paraffin-embedded cases this fiscal

year and is recognized as a national leader in this area. Basic research includes transposable elements in cancer, molecular pathobiology of breast cancer, transcriptional regulation in viruses, tumor suppressor gene function, and development of the hematopoietic system. Applied research ranges from the molecular epidemiology of morbilliviruses in dolphins to the diagnosis of the (2;5) translocation in anaplastic large cell lymphoma. This division accounts for the bulk of the department's grant funding, which comes from such diverse sources as the Army Medical Research and Development Command, the National Institutes of Health, and others. Dr. Taubenberger also serves as an attending pathologist at the National Institutes of Health.

OTHER INTERESTING FACTS

Department staff (average age < 40) has published over 250 refereed papers.

DIVISION OF BIOPHYSICS

Jeffrey T. Mason, Ph.D.
Chief

MISSION

The mission of the division is to develop new knowledge and techniques in basic and applied molecular biology through the application of biochemical, biophysical, and chemical methods to the study of biological systems. Techniques utilized include flow cytometry, organic synthesis, infrared and Raman spectroscopy, scanning and titration calorimetry, fluorescence spectroscopy, x-ray diffractometry, and atomic force and electron microscopy.

ORGANIZATION

STAFF

Scientific

(A) Michael M. Batenjany, Ph.D.
Robert E. Cunningham, M.S.
Jeffrey T. Mason, Ph.D.
Timothy J. O'Leary, M.D., Ph.D.

RESEARCH

Research activities during the past year include developmental work on immunoliposome-PCR (an ultrasensitive assay system for biological and chemical antigens); continuing studies designed to understand the structure and function of biological membranes and the interaction of these systems with extrinsic molecules, such as alcohols and anesthetics; and the influence of alterations of membrane lipid structure and pressure on the function of integral membrane proteins, such as acetylcho-

EXHIBIT 5

20 April 2001

Mr. Scott Glisson
U.S. Army Medical Research and Material Command
ATT: MCMR-AAA (PRMRP-01)
820 Chandler Street
Fort Detrick, MD 21702-5014

Subject: PRMRP-LOI
Topic Area: Biological Hazard Detection Systems
Principal Investigator: Jeffrey T. Mason, PhD
Submitting Organization: American Registry of Pathology

Dear Mr. Glisson:

This letter is to serve as a letter-of-intent to submit a proposal to the peer reviewed medical research program (PRMRP) supplement to the USAMRMC under the FY01 PRMRP topic area of "biological hazard detection systems". Our proposal will describe an extremely sensitive, but simple, method to detect biological hazards at levels approaching fewer than 100 molecules. This method couples the selectivity and high binding affinity of antibodies for biological molecules with the near-infinite signal amplification capability of PCR. This will be accomplished by encapsulating short DNA sequences (primers) for PCR amplification into enclosed phospholipid membranes (liposomes) and conjugating antibodies specific to biological hazardous (such as botulinum toxin) to the outer liposomal membranes. Extremely low numbers of toxin molecules can then be detected by employing a hybrid ELISA-PCR assay.

The Principal Investigator will be:

Jeffrey T. Mason, PhD
Chief, Division of Biophysics
Department of Cellular Pathology
Armed Forces Institute of Pathology
Rockville Annex
Room 1057D, Building #101
1413 Research Boulevard
Rockville, MD 20850

Phone: 301-319-0043
Fax: 301-319-0638
E:mail mason@afip.osd.mil

Collaborators will include Timothy J. O'Leary, MD, PhD, Chairman, Department of Cellular Pathology, AFIP and 1-2 postdoctoral fellows (TBA) to be employed through the American Registry of Pathology. We thank you in advance for your consideration of our application.

Sincerely yours,

Jeffrey T. Mason, PhD

EXHIBIT 6

BAA 99-1 RESEARCH PROPOSAL COVER PAGE

1. USAMRMC Log No.:	USAMRMC PROPOSAL COVER PAGE
2. Name and Address of Offeror: American Registry of Pathology Building #102, Room 2215 1413 Research Boulevard Rockville, MD 20850	3. Type of Organization: ☐ EDUCATIONAL: ☐ HBCU ☐ MI ☐ FDP ☐ COMMERCIAL: ☐ Large ☐ Small ☐ Woman-Owned ☐ Disadvantaged Business ☒ NON-PROFIT
4. Data Universal Numbering System (DUNS): 114 400 633	5. Trading Partner Identification No. (TPIN): Not Applicable
6. Standard Industrial Classifications (SIC): 8701	7. Federal Supply Classifications (FSC): Not applicable
7. Commercial and Gov't Entity (CAGE): 2U804	8. Taxpayer Identification No. (TIN): 52-1073470
9. Federal Interagency Committee on Education (FICE) No.: Not applicable	
11. Proposal Title: A field-deployable ultra-sensitive assay system for biological toxins using immunoliposome – DNA amplification hybrids	
12. Estimated Cost: $382,691	13. Proposed Start Date: 01/01/2002
14. Proposed Duration 3 years	15. Proposal Valid Until: 01/01/2002
16. Principal Investigator's Name and Address: Jeffrey T. Mason, PhD Chief, Division of Biophysics Building #101, Room 1057D Armed Forces Institute of Pathology 1413 Research Boulevard Rockville, MD 20850	17. Administrative Representative Name & Address: Michael D. Parry Director of Operations American Registry of Pathology Building #102, Room 2219 1413 Research Boulevard Rockville, MD 20850
Email: mason@afip.osd.mil	Email: parry@afip.osd.mil
Phone No.: 301-319-0043	Phone No.: 301-319-0087
FAX No: 301-319-0638	FAX No: 301-319-3549
Alternate's Name: Timothy J. O'Leary, MD, PhD	Alternate's Name: Eliana Andrea
Alternate's Phone No: 301-319-0149	Alternate's Phone No: 301-319-0090
18. Authorized Representative:	
Typed Name: Michael D. Parry	Signature: Michael D Parry
Title: Director of Operations	Date Signed: 5/14/01

NOTHING ON THIS PAGE IS PROPRIETARY INFORMATION

BAA 99-1 PROPOSAL ABSTRACT

Proposal Title: *(120 Characters Maximum)*

A Field-Deployable Ultra-sensitive Assay System for Biological Toxins using Immunoliposome-DNA Amplification Hybrids (Jeffrey T. Mason, submitting investigator)

Keywords: *(6-8 words)*

Biotoxins, Immunoassay, Liposome, Antibody, Tetanus, Botulinum, PCR

Abstract: (Type within outline: approximately 200 words)

Background: The early and rapid detection of biological toxins is critically important to the protection of military personnel deployed in hostile peacekeeping or combat situations. Biological toxins, such as botulinum toxin, are lethal at extremely low concentrations, which necessitates detection methods that are both highly specific and extremely sensitive. Current methods for detecting biological toxins, such as immunoassays, mass spectrometry, and DNA amplification techniques, are not well suited to the development of highly specific assay systems for detecting biological toxins at attomolar concentrations in military deployment situations.

Objective: The objective of this proposal is the development of a simple and reliable field-deployable assay system for the detection of biological toxins with high specificity and at attomolar concentrations using immunoliposome-DNA amplification hybrids. DNA templates (amplicons) will be encapsulated inside single-bilayer closed-shell liposomes. Antibodies directed against Botulinum or Tetanus toxoid will then be covalently attached to the extravesicular surface of the liposomes to form what we refer to as DNA-competent immunoliposomes. These liposomes will be allowed to bind to toxoid that has been adsorbed inside the wells of a micro-titer plate. Following removal of unbound liposomes, the bilayers will be ruptured with a nonionic detergent to release the amplicons, which will then be amplified by PCR and detected by gel or capillary electrophoresis. This unique protein assay combines the specificity of an immunoassay with the dramatic detection amplification capability of PCR.

Specific Aims: We propose, in this application, to accomplish the following tasks:
1. To develop a method to produce, store, and characterize DNA-competent immunoliposomes.
2. To develop and optimize immunoassay conditions for the detection of botulinum and tetanus toxoid using DNA-competent immunoliposomes and to determine the lower limit of detection of these toxoids using this technique.
3. To further develop the assay system so that multiple biological toxins can be detected in a single assay by associating each antibody with liposome-encapsulated templates of a unique length.
4. To determine the optimal immunoliposome composition and immunoassay conditions necessary to minimize non-specific binding of immunoliposomes during the assay.
5. To optimize detection of the PCR-amplified DNA by gel and capillary electrophoresis.
6. To develop and test a simple field-deployable version of the assay.

Study Design: The specific aims of this proposal will be achieved through the following sequence of steps: producing amplicons for encapsulation, perfecting methods to encapsulate amplicons in liposomes, perfecting methods to covalently attach antibodies to the extravesicular surface of the liposomes, perfecting methods to purify the DNA-competent immunoliposomes, developing methods to store and characterize DNA-competent immunoliposomes, developing and perfecting immunoassay conditions for detecting toxoid with DNA-competent immunoliposomes, establishing optimal conditions for minimizing non-specific binding of liposomes during the immunoassay, optimizing conditions for detecting the PCR amplified amplicons by gel and capillary electrophoresis, and developing and testing a field-deployable version of the assay system.

Military Relevance: The ability to detect biological toxins at extremely low concentration levels will increase the time for troops in the field to undertake countermeasures, thus decreasing the likelihood of death or injury due to exposure. Military intelligence activities aimed at the detection of suspected sites of manufacturer or storage of biological toxins and the analysis of trace forensic evidence from terrorist activities would also benefit from the ability ot detect biological toxins with high specificity and at extremely low concentration levels.

NOTHING ON THIS PAGE IS PROPRIETARY INFORMATION

EXHIBIT 7

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA

INVENTION DISCLOSURE

(THIS FORM AND ACCOMPANYING DRAWING AND DESCRIPTION SHEETS ARE TO BE COMPLETED FOR EACH INVENTION PROMPTLY FORWARDED TO THE PATENT ACTIVITY*)

PATENT ACTIVITIES DOCKET NO

ASSIGNED TO:

SHORT TITLE OF INVENTION
Immunoliposome-Nucleic Acid Amplification Assay Method

FULL NAME(S) OF INVENTOR(S) (FIRST) (MIDDLE INITIAL) (LAST)	HOME ADDRESS(ES)	(DUTY) TEL. NO. AREA CODE
(1) Timothy J. O'Leary	703 Hermleigh Rd., Silver Spring, MD 20902	301-319-0202
(2) Jeffrey T. Mason	19605 Ridge Heights Dr., Gaithersburg, MD 20879	301-319-0043
(3)		

INFORMATION AND DATES CONCERNING THIS INVENTION

NEEDED IN THE EVENT OF A CONTEST OF PRIORITY OF INVENTION IN THE U.S. PATENT AND TRADEMARK OFFICE, ALL RECORDS CITED SHOULD BE DATED AND SIGNED BY TWO INDEPENDENT WITNESSES WHO HAVE READ AND UNDERSTOOD THE MATERIAL.

ON WHAT DATE DID YOU FIRST THINK OF THIS INVENTION (WHAT RECORDS SHOW THIS?)
(4)

GIVE DATE OF AND IDENTIFY EARLIEST SKETCH OR DRAWING
(5)

WHEN/WHERE AND TO WHOM DID YOU MAKE THE FIRST DISCLOSURE TO OTHERS OF THE INVENTION EITHER ORALLY OR IN WRITING?
(6)

DESCRIBE DETAILS OF ANY WORK OR TESTS DONE TO PRODUCE OR OPERATE THE INVENTION GIVE DATES AND WITNESSES (USE OTHER PAGES IF NECESSARY)
(7)

DESCRIBE AND GIVE DATES OF ANY OTHER SKETCHES, DRAWINGS, OR REPORTS PERTINENT TO THIS INVENTION
(8) None

USE, SALE OR PUBLICATION

NEEDED TO ESTABLISH THE DATE OF ANY PRINTED PUBLICATION, PUBLIC USE OR SALE. SINCE NO PATENT APPLICATION MAY BE FILLED AFTER ONE YEAR FROM SUCH DATE.

IF INVENTION HAS BEEN SOLD OR USED FOR PROFIT-WHEN AND TO WHOM DISCLOSED OR WHEN AND HOW USED?
(9) N/A

HAS A DESCRIPTION OF THIS INVENTION BEEN MADE AVAILABLE TO PERSONS OUTSIDE THE ARMY? (WRITTEN OR ORAL) IF SO, HOW AND WHEN AND WAS USE RESTRICTED?
(10) No

POTENTIAL MARKET INFORMATION

NEEDED FOR POSSIBLE MARKETING INVESTIGATIONS AND AS AN AID TO POTENTIAL LICENSING TO OTHERS.

DESCRIBE ANY POTENTIAL, OR EXISTING MARKET FOR SALE OR LICENSE OF THIS INVENTION
(11)
A. GOVERNMENT:
B. COMMERCIAL: Please see Appendix IV
C. IDENTIFY ANY KNOWN FIRMS OR VENDORS WHO MAY BE INTERESTED IN THE INVENTION:

CONTRACT INFORMATION

A DETERMINATION OF RIGHTS IN THIS INVENTION WILL BE NECESSARY. (SEE AR 27-60)

IF THIS INVENTION WAS FIRST CONCEIVED OR CONSTRUCTED IN CONNECTION WITH:
(12)
(A.) MY DUTIES AS A GOVERNMENT EMPLOYEE
B. MY WORK UNRELATED TO MY DUTIES AS A GOVERNMENT EMPLOYEE (PRIVATE, OFF DUTY ACTIVITIES)
C. MY DUTIES AS A GOVERNMENT EMPLOYEE & WORKING WITH A CONTRACTOR
D. NEITHER A, B , OR C, EXPLAIN

FOREIGN FILING CONSIDERATION

NEEDED TO DETERMINE THE POTENTIAL WORLDWIDE USE FOR THE INVENTION.

INDICATE THE POTENTIAL FOR USING THIS INVENTION IN FOREIGN COUNTRIES
(13)
☐ POOR
☐ GOOD
☑ EXCELLENT

SECURITY CLASSIFICATION

PLEASE INDICATE THE SECURITY CLASSIFICATION IF KNOWN
(13A)
☐ CLASSIFIED
LEVEL
☑ UNCLASSIFIED
☐ CLASSIFICATION UNKNOWN

DA FORM 4734-R, 1 OCT 1978 USAPA V1.00

DEPARTMENT OF THE ARMY
UNITED STATES OF AMERICA
INVENTION DISCLOSURE

(DRAWING AND DESCRIPTION SHEET)

PATENT ACTIVITIES DOCKET NO

(14) PROVIDE THE FOLLOWING INFORMATION CONCERNING THE DISCLOSED INVENTION AND IN THE INDICATED SEQUENCE:

A. SPECIFICALLY DESCRIBE THE INVENTION AND ITS OPERATION. YOU MAY USE AND ATTACH COPIES OF SKETCHES, PRINTS, PHOTOGRAPHS, PAPERS AND ILLUSTRATIONS, WHICH SHOULD BE SIGNED, WITNESSED, AND DATED. USE NUMBERS AND DESCRIPTIVE NAMES IN DESCRIPTIONS AND DRAWINGS.

B. STATE THE ADVANTAGES OF THE INVENTION OVER PRESENTLY KNOWN DEVICES, SYSTEMS, OR PROCESSES.

C. DISCUSS THE PROBLEMS WHICH THE INVENTION IS DESIGNED TO SOLVE, REFERRING TO ANY PRIOR INVENTION OF A SIMILAR NATURE WITH WHICH YOU MAY BE FAMILIAR.

D. LIST ALL KNOWN AND OTHER POSSIBLE USES FOR THE INVENTION.

E. LIST THE FEATURES OF THE INVENTION THAT ARE BELIEVED TO BE NOVEL.

USE AS MANY OF THESE SHEETS AS NECESSARY AND ATTACH TO COMPLETED INVENTION DISCLOSURE

PLEASE SEE ATTACHED SHEETS FOR:

1. Section 14 (A-E)
2. Appendix I (Detailed Methods)
3. Appendix II (Literature References)
4. Appendix III (Results of Patent Search)
5. Appendix IV (Section 11, Potential Market Information)

SIGNATURE(S) AND ORGANIZATION OF INVENTOR(S) (USE INK) DATE: THE DESCRIBED INVENTION HAS BEEN WITNESSED READ AND UNDERSTOOD BY: DATE:

(15) [signature] 11/4/02 (18) [signature] 10 JAN 03

ORGANIZATION Armed Forces Institute of Pathology

(16) [signature] 11/4/02 (19) _____

ORGANIZATION Armed Forces Institute of Pathology

(17) _____ (20) _____

ORGANIZATION _____

*NOTE: THIS FORM AND ANY OMITTED INFORMATION BECOMING AVAILABLE AT A LATER TIME SHOULD BE FORWARD TO:
HQDA CHIEF, INTELLECTUAL, PROPERTY DIV. OFFICE OF THE JUDGE ADVOCATE GENERAL DEPT. OF THE ARMY WASHINGTON, D.C., 20310
DARCOM ATTN: PATENT COUNSEL; OR CHIEF OF ENGINEERS ATTN: PATENT COUNSEL

(14) PROVIDE THE FOLLOWING INFORMATION CONCERNING THE DISCLOSED INVENTION AND IN THE INDICATED SEQUENCE

A. SPECIFICALLY DESCRIBE THE INVENTION AND ITS OPERATION

I. NON-TECHNICAL DESCRIPTION OF THE INVENTION

This invention is a method for detecting extremely small quantities (as few as 10-1000 molecules) of compounds for which specific receptors (such as antibodies) exist. The receptors are coupled to the outer surface of closed-shell bilayer membranes (liposomes) that are formed in such a way that they encapsulate 100-1000 copies of short DNA or RNA segments (amplification substrates) that are capable of being amplified by the polymerase chain reaction (PCR) or any other nucleic acid amplification technology (such as RT-PCR, bDNA or Q-beta-replicase). As the receptors used are typically antibodies these structures are referred to as "immunoliposomes", which are depicted in Figure 1. The target antigen is immobilized on a substrate (typically inside the wells of a microtiter plate) and unbound antigen is removed by rinsing. The immunoliposomes are then added to the solution and allowed to bind to the target antigen and any unbound immunoliposomes are subsequently removed by rinsing. The remaining specifically bound immunoliposomes are ruptured by the addition of a non-ionic detergent, which causes them to release their amplification substrates into the bulk solution. The amplification substrates are then amplified by PCR or RT-PCR and detected by gel or capillary electrophoresis. Alternately, the released amplification substrates can be amplified and detected by a real-time (homogeneous) PCR/RT-PCR assay or any other coupled nucleic acid amplification/detection technology (such as bDNA or Q-beta-replicase). We refer to this invention as the immunoliposome-nucleic acid amplification (ILNAA) assay method, which is depicted diagrammatically in Figures 1 and 2(A-C).

II. MILITARY RELEVANCE

The early and rapid detection of biological toxins is critically important to the protection of military personnel deployed in hostile peacekeeping or combat situations. Biological toxins, such as botulinum toxin, are lethal at very low concentrations, which necessitates detection measures that are both highly specific and extremely sensitive. There are a multitude of military scenarios that may require the ability to detect biological toxins at sub-attomolar (10^{-18}M) concentrations or even at levels approaching a few molecules. Foremost, early detection of the use of biological toxins by the enemy will allow time for troops in the field to undertake countermeasures, thus decreasing the likelihood of death or injury due to exposure. Military intelligence activities frequently require attempts to identify sites of manufacture or storage of biological toxins by soil or water sampling at considerable distances from the suspected site. Intelligence activities may also include attempts to identify former storage sites for biological weapons after the enemy has moved the material and attempted to sterilize the area. The military is frequently called upon to clean up storage sites for biological weapons, which requires the ability to survey for residual contamination. Finally, terrorist acts involving biological toxins will frequently require examination of trace forensic evidence for the presence of toxins. All of these examples point to the need for rapid and reliable tests for biological toxins that are highly specific, but also sufficiently sensitive to detect the target toxin down to the level of a few molecules. A summary of the potential military applications of this invention are:

1. The detection of biological toxins
2. The detection of infectious bacteria and viruses
3. The detection of chemical warfare agents
4. The detection of poisons
5. The detection of explosive compounds
6. The detection of trace forensic evidence

Inventor Tanjore Z Date 1/10/03 Witness [signature] Date 1/10/03

Inventor [signature] Date 1/10/03 Witness _____ Date _____

IMMUNOLIPOSOMES SHOWN BOUND TO ANTIGEN (BIOTOXIN) IN AN IMMUNOASSAY WITH DIRECT OR INDIRECT BINDING OF ANTIGEN TO THE MICROTITER PLATE SUBSTRATE

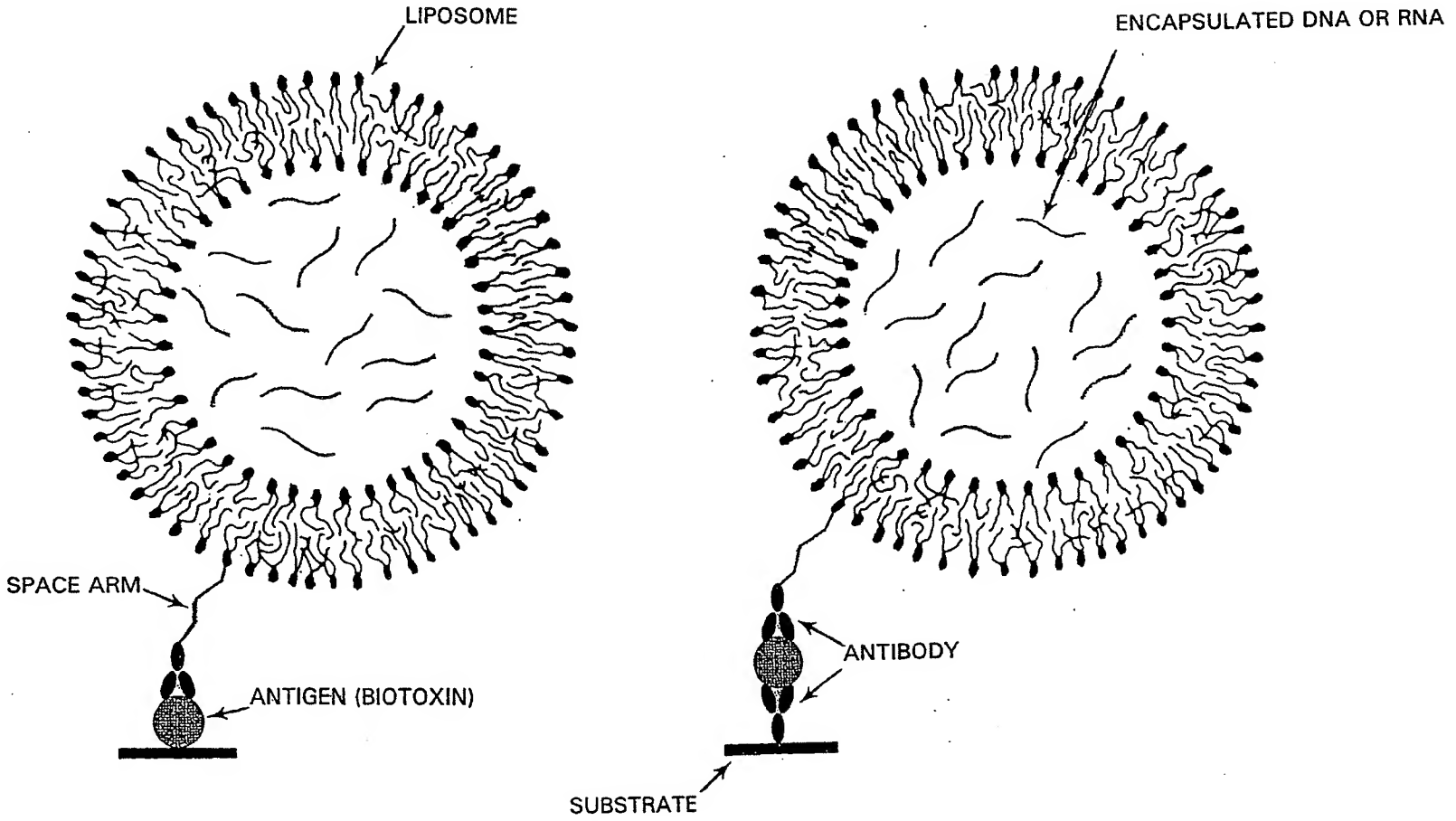


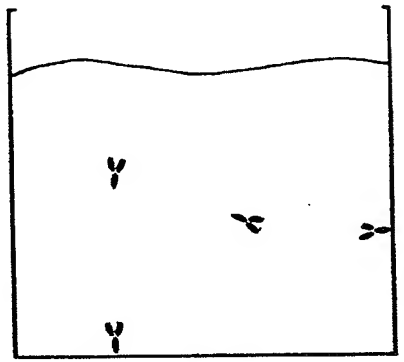
FIGURE 1

IMMUNOLIPOSOME NUCLEIC ACID AMPLIFICATION ASSAY METHOD

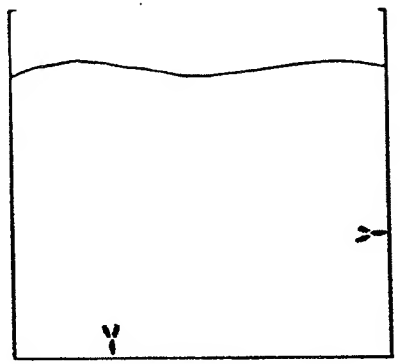
Inventor [signature] Date 1/10/03 Witness [signature] Date 1/10/03

Inventor [signature] Date 1/10/03 Witness _____ Date _____

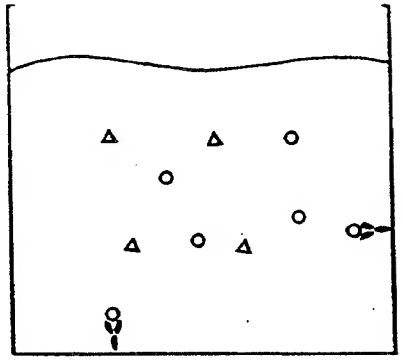
INDIVIDUAL STEPS OF AN INDIRECT ILNAA ASSAY FOR A BIOTOXIN ANTIGEN



Add primary antibodies and allow them to bind to the well



Rinse free antibodies leaving specifically bound antibodies



Add sample containing Biotoxin, allow it to bind to the antibodies

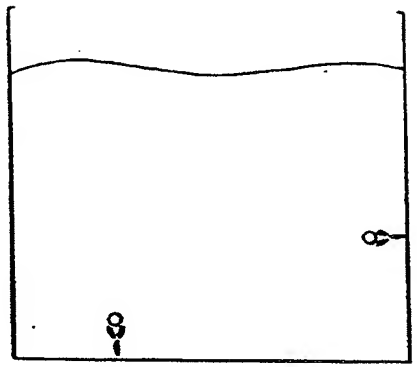
FIGURE 2A

IMMUNOLIPOSOME NUCLEIC ACID AMPLIFICATION ASSAY METHOD

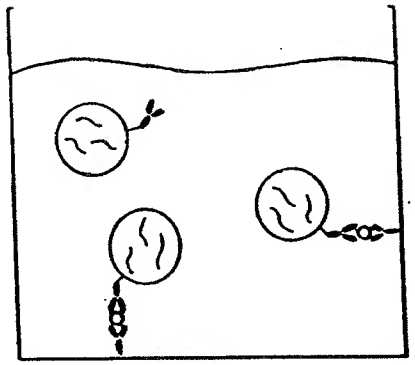
Inventor _____ Date 1/10/03 Witness _____ Date 1/10/03

Inventor _____ Date 1/9/03 Witness _____ Date _____

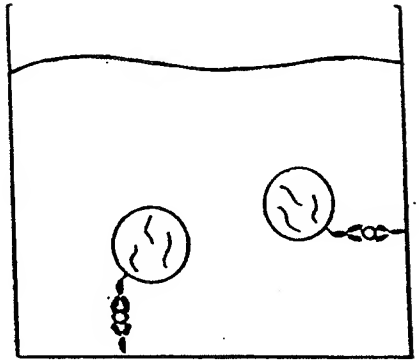
INDIVIDUAL STEPS OF AN INDIRECT ILNAA ASSAY FOR A BIOTOXIN ANTIGEN



Rinse free antigens leaving only specifically-bound target Biotoxin



Add immunoliposomes, allow to bind to target Biotoxin



Rinse free immunoliposomes leaving specifically-bound immunoliposomes

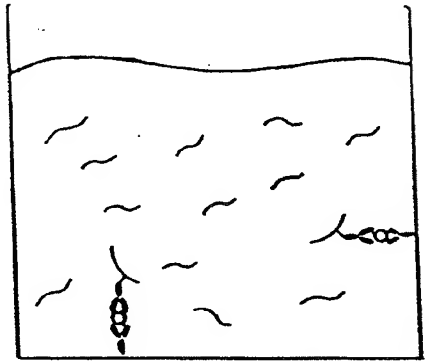
FIGURE 2B

IMMUNOLIPOSOME NUCLEIC ACID AMPLIFICATION ASSAY METHOD

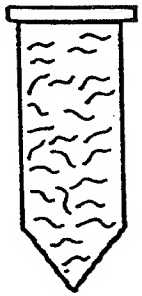
Inventor [signature] Date 11/06/3 Witness [signature] Date 1/10/03

Inventor [signature] Date 11/4/03 Witness _____ Date _____

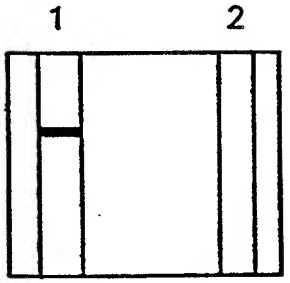
INDIVIDUAL STEPS OF AN INDIRECT ILNAA ASSAY FOR A BIOTOXIN ANTIGEN



Rupture the immunoliposomes with detergent and release the entrapped DNA



Amplify the released DNA by multiple PCR cycles



Perform gel electrophoresis to detect the presence of Biotoxin (lane 1) versus its absence in a control sample (lane 2)

FIGURE 2C

IMMUNOLIPOSOME NUCLEIC ACID AMPLIFICATION ASSAY METHOD

III. TECHNICAL DESCRIPTION OF THE INVENTION

The primary intent of the ILNAA assay method (the invention) was to design a highly specific and extremely sensitive assay to detect biological toxins used as bio-warfare agents. However, the ILNAA assay method has been designed to be as versatile as possible so that the assay can be used to detect virtually any compound for which a specific receptor exists. Accordingly, the technical description is divided into three sections. The first section (A) describes the key elements of the assay with particular emphasis on the variable elements of the assay whose choice depends upon the target compound to be detected and the method to be used to detect the amplified nucleic acid product (such as a PCR product). The second section (B) describes the entire ILNAA assay method in detail by using the detection of cholera toxin as a specific example. The third section (which is included as appendix I) provides detailed information on all of the individual steps of the ILNAA assay method with information on alternate approaches for optimizing the assay and quality control procedures. See appendix II for all literature references. See appendix III for the results of a patent search of related methodologies.

A. Key Elements of the ILNAA Assay Method

1) *Choice of a specific receptor molecule*: By specific receptor molecule we refer to a molecule that serves to unambiguously identify a compound (or class of compounds) by tightly and specifically binding to (or reacting with) that compound and no other. The choice of the specific receptor molecule is dependent upon the compound that one desires to detect. In most cases this will be a protein and the specific receptor will be a monoclonal or polyclonal antibody directed against that protein. However, other specific receptor molecules that can be employed include (but are not limited to) gangliosides, glycolipids, biological membrane receptors, molecules that bind specific ions, and compounds designed to bind specifically to (or react specifically with) chemical warfare agents, explosives, poisons, or any other molecule. Also included are soluble proteins, dyes, and DNA or RNA probes designed to bind to specific nucleic acid target sequences.

2) *Choice of Amplification substrates*: An amplification substrate is a short DNA or RNA segment that can be amplified by PCR, RT-PCR, or any other nucleic acid amplification process. As the amplification substrate serves only as a signal amplification mechanism the choice of the amplification substrate to use is not critical. However, certain guidelines are that the amplification substrate is short (25-100 base pairs) to maximize the number of amplification substrates that can be encapsulated per liposome and that the amplification substrate is not likely to be found in the sample being analyzed in order to reduce the likelihood of false positives.

3) *Choice of Encapsulation Method*: In the context of this invention "liposome" refers to any structure that can (a) encapsulate amplification substrates thereby sequestering them from the chemical environment outside of the structure, (b) allow for the attachment of the chosen specific receptor molecule to the outer surface of the structure, and (c) allow for the release (by any method) of the encapsulated amplification substrates into the bulk solution for nucleic acid amplification and detection after specific binding to the target compound has been achieved. In our invention we typically employ closed-shell phospholipid bilayers as the encapsulating structure, however, any other suitable encapsulating structure could be employed.

4) *Choice of Coupling Method*: The coupling method refers to the process whereby the chosen specific receptor molecule is attached to (or incorporated into) the outer surface of the encapsulating structure. The coupling method to be employed will depend upon the chemical properties of both the specific receptor molecule chosen and the encapsulating structure to be employed. The coupling can be either covalent or non-covalent in nature.

5) *Choice of Nucleic Acid Amplification Method:* The nucleic acid amplification method refers to the process by which the chosen amplification substrate is reproduced for detection. The amplification method to be employed (such as PCR or RT-PCR) will depend upon the amplification substrate that has been chosen, the analyte of interest, and the conditions under